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Adenosine A_{2A} receptors assemble into higher-order oligomers at the plasma membrane

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Abstract Oligomerization of G protein-coupled receptors (GPCRs) is known to play important roles in regulating receptor pharmacology and function. Whereas many bivalent GPCR interactions have been described, the stoichiometry and localization of GPCR oligomers are largely unknown. We have used bimolecular fluorescence complementation (BiFC) to study adenosine A_{2A} receptor ($A_{2A}R$) oligomerization. The data suggest specificity of the $A_{2A}R/A_{2A}R$ interaction monitored by BiFC and proper sub-cellular localization of tagged receptors. Moreover, using a novel approach combining fluorescence resonance energy transfer and BiFC, we found that at least three A_{2A} receptors assemble into higher-order oligomers at the plasma membrane in Cath.A differentiated neuronal cells.

Structured summary:
MINT-6797156, MINT-6797142:
A2AR (uniprotkb:P29274) *physically interacts* (MI:0218) with *A2AR* (uniprotkb:P29274) by *bimolecular fluorescence complementation* (MI:0809)
MINT-6797129:
A2AR (uniprotkb:P29274) *physically interacts* (MI:0218) with *A2AR* (uniprotkb:P29274) by *fluorescent resonance energy transfer* (MI:0055)

Keywords: Bimolecular fluorescence complementation; Fluorescence (Forster) resonance energy transfer; Adenosine A_{2A} receptor; G protein-coupled receptor; Higher-order oligomer; Neuronal cell

1. Introduction

With approximately 1000 members, the GPCR superfamily is the largest class of membrane receptors in mammals, serving as targets for many therapeutic drugs. A growing number of GPCRs are known to exist as dimers. Although several GPCRs may be able to signal to their effectors as monomers [1,2], dimerization often confers unique functional properties and/or pharmacological profiles to receptors, and may hence represent an important regulatory mechanism for GPCR function [3–6].

The adenosine A_{2A} receptors are highly expressed on striatal spiny neurons [7] where they positively regulate adenylyl cyclase (AC) activity through coupling to $G\alpha_{s/olf}$. $A_{2A}R$ homodimer [8–10], as well as heterodimer complexes, notably with dopamine D_2 receptors (D_2R) [3,11–13], have been reported. $A_{2A}R$ (as well as other GPCRs), may further associate as higher-order oligomers in signaling complexes. Consistent with this hypothesis is the observation that $A_{2A}R/D_2R$ heterodimers colocalize with $A_{2A}R/A_{2A}R$ homodimers [14]. To examine the possibility of higher-order complex formation, we have used a combination of bimolecular fluorescence complementation (BiFC) [15] and fluorescence (Forster) resonance energy transfer (FRET) techniques in Cath.A differentiated (CAD) neuronal cells. The results indicate that A_{2A} receptors associate in higher-order oligomers at the plasma membrane.

2. Materials and methods

2.1. Materials

$A_{2A}R$, M_4R and D_1R cDNAs were obtained from the Missouri S&T cDNA Resource Center. Growth media and reagents (unless otherwise stated) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Expression vectors

Human $A_{2A}R$ and D_1R coding sequences were cloned in pBiFC vectors [16] to generate C-terminal fusions with fragments from Venus or Cerulean fluorescent proteins, as described [14]. N-terminal fragments from Venus or Cerulean (VN or CN) encompass residues 1–172 whereas C-terminal fragments (VC or CC) consist of residues 155–238. To generate VN and VC fusions to the human muscarinic M_4 receptor, M_4R coding sequence was amplified by polymerase chain reaction using 5′ cgg aat tct tAT GGC CAA CTT CAC ACC TGT C 3′ and 5′ cgc tcg agc CCT GGC AGT GCC GAT GTT C 3′oligonucleotides, and inserted between the EcoRI and XhoI sites from pBiFC-VN or pBiFC-VC. The M_4R-Venus construct was obtained by swapping VN with the Venus coding sequence. The constructs were verified by DNA sequencing.

2.3. Cell culture and transient gene expression

CAD cells were maintained as described previously [17]. Cells were grown to 70% confluency in 12-well plates for whole cell fluorescence measurements or in four-well Lab-Tek chambered coverslips (Nunc, Rochester, NY) for microscopy. Cells were transfected using 2 μl (12-well plates) or 1 μl (coverslips) Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. DNA amounts per well in 12-well plates were 300 ng ($A_{2A}R$, D_1R,

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or M_4R), or 200 ng (Cerulean). DNA amounts per well in coverslips were 100 ng ($A_{2A}R$), 200 ng (M_4R), or 20 ng (mCherry-Mem, YFP-Endo, YFP-Golgi, and YFP-ER).

2.4. Fluorescence measurement in cell suspensions

CAD cells were suspended in PBS and transferred into 96-well plates (Nunc; 40 μg protein/well). Protein concentration was determined using the BCA method (Pierce, Rockford, IL). Cerulean and Venus fluorescence were measured with a multi-well plate reader (FUSION Packard, Waltham, MA) using 430/25 nm and 500/20 nm excitation, as well as 470/30 nm and 535/30 nm emission filters, respectively. Mock-transfected cells were used for background subtraction. BiFC signals were normalized with signals from co-transfected Cerulean. FRET signals (F) were measured using the sensitized acceptor method. For each sample, Cerulean (C) and Venus (V) fluorescence was measured as above. In addition, FRET signals were collected using 430/25 nm excitation and 535/30 nm emission filters. Acceptor bleed-through (a = F/V) and donor cross-talk (d = F/C) coefficients were determined with cells expressing either Venus or Cerulean fusion proteins. Corrected FRET signals (cFRET) were calculated according to the equation

$$\mathrm{cFRET} = F - aV - dC$$

and normalized with donor (C) and acceptor (V) fluorescence intensities as

$$\mathrm{N\ FRET} = \mathrm{cFRET}/\sqrt{(C \times V)}$$

2.5. Microscopy

Twenty four hours post-transfection, the growth medium was replaced with PBS and cells were imaged using a charge-coupled device camera mounted on a TE2000-U inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a 100 W mercury lamp and band-pass filters (Chroma, Rockingham, VT) for Venus (500/20 nm excitation, 535/30 nm emission), Cerulean (430/25 nm excitation, 470/30 nm emission) or mCherry (572/23 nm excitation). Fluorescent images were acquired using the MetaMorph software (Molecular Devices, Sunnyvale, CA) and images were analyzed using ImageJ (http://rsbweb.nih.gov/ij/) as previously described [14].

2.6. FLIM analysis

Fluorescence lifetime measurements were performed with a confocal setup using an inverted Olympus IX71 microscope (Center Valley, PA) equipped with a picosecond pulsed diode laser (PicoQuant GmbH, Berlin, Germany) with emission at 467 nm as excitation source. The laser power used in the experiments was 3 μW and the pulse frequency of 40 MHz. The laser beam was focused in the sample volume using an apochromatic 60× water immersion objective of numerical aperture 1.2 and the emitted fluorescence was collected using the same objective and separated from excitation beam by a dichroic mirror. A 50 μm pinhole was used to reject off-focus fluorescence from the excitation volume. To ensure photon counting emitted by the donor fluorochrome only, a band-pass filter (475–485 nm, Omega Optical, Brattleboro, VT) was placed in front of the single photon avalanche photodiodes (SPAD; SPCM-AQR, Perkin–Elmer Inc., Waltham, MA). The fluorescence was measured using time correlated single photon counting (TCSPC) in time tagged time resolved (TTTR) mode (Time Harp200, PicoQuant). To obtain fluorescence lifetimes, TCSPC decay curves were fitted by double exponential using the SymphoTime software (PicoQuant). FRET efficiencies (E) were calculated using the equation:

$$E = 1 - (\tau DA/\tau D)$$

with τDA and τD the donor excited state lifetime in the presence and absence of acceptor, respectively.

2.7. Cyclic AMP accumulation assays

Cyclic AMP measurements were performed in HEK293 cells as described [14] in order to verify M_4R function. Forskolin (30 μM) was used to stimulate adenylyl cyclase. Carbachol (1 μM) and atropine (10 μM) were used as M_4R agonist and antagonist, respectively. Stimulations were performed at 37 °C for 5 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 500 μM).

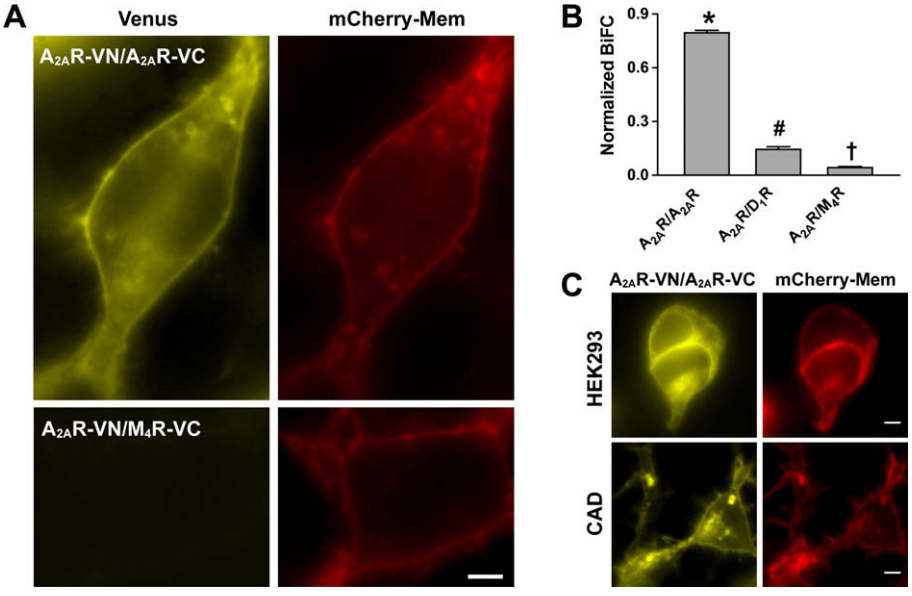

Fig. 1. $A_{2A}R$ oligomerization detected with BiFC. (A) Signals from Venus complementation (yellow) in cells transfected with $A_{2A}R$-VN and $A_{2A}R$-VC or M_4R-VC, as well as signals from the co-transfected membrane marker mCherry-Mem, were monitored by fluorescence microscopy. (B) Fluorescence intensity in cells expressing $A_{2A}R$-VN and $A_{2A}R$-VC, D_1R-VC, or M_4R-VC was quantified by fluorometry. BiFC signals were normalized with co-transfected Cerulean. Data are means ± S.E.M. from three independent experiments assayed in triplicate. Different symbols indicate significant differences ($P < 0.001$; One-way ANOVA followed by Bonferroni's post hoc test). (C) $A_{2A}R$-VN and $A_{2A}R$-VC fusions were expressed in HEK293 or CAD cells. Fluorescence complementation, as well as signals from co-transfected mCherry-Mem were detected by fluorescence microscopy. *Scale bars:* 5 μm.

3. Results

3.1. $A_{2A}R$ dimers detected by BiFC in CAD neuronal cells

We chose to study $A_{2A}R$ oligomerization in the CAD neuronal cell line that endogenously express adenosine and dopamine receptors [18–20]. $A_{2A}R$ fusions to N- and C-terminal portions of the cyan fluorescent protein Cerulean ($A_{2A}R$-CN and $A_{2A}R$-CC) or the yellow fluorescent protein Venus ($A_{2A}R$-VN and $A_{2A}R$-VC) were transiently expressed in CAD cells. The C-terminal tagged A_{2A} receptors retained the ability to bind [^{3}H] ZM 241–385 and to stimulate adenylyl cyclase (AC) through coupling to $G\alpha_s$ ([14] and data not shown). C-Terminal BiFC fusions to the muscarinic M_4 receptor (M_4R) and to the dopamine D_1 receptor (D_1R) that were used in control experiments also retained the ability to regulate AC ([14] and Supplementary Fig. S1). Therefore, the BiFC tags had no detrimental effect on receptor function.

Co-expression of $A_{2A}R$-VN and $A_{2A}R$-VC in CAD or HEK293 cells resulted in robust bimolecular fluorescence complementation (Fig. 1), indicating $A_{2A}R$ dimerization and consistent with previous studies [21,22]. $A_{2A}R$-VN/$A_{2A}R$-VC signals largely overlapped at the cell surface with the membrane marker mCherry-Mem (see below). No fluorescence was observed in cells expressing only VN or VC fragments (data not shown). Normalized BiFC signals in cells expressing $A_{2A}R$-VN and $A_{2A}R$-VC (6.57±0.57; $n = 49$) were markedly higher than in control transfections with $A_{2A}R$-VN and M_4R-VC (0.48±0.06; $n = 74$) (Fig. 1A). Moreover, whole cell fluorescence intensity in $A_{2A}R$-VN/$A_{2A}R$-VC-transfected cells was much stronger than in control transfections with $A_{2A}R$-VN/D_1R-VC or $A_{2A}R$-VN/M_4R-VC (Fig. 1B), indicating specificity of the measured $A_{2A}R/A_{2A}R$ interaction.

3.2. Sub-cellular localization of $A_{2A}R$ dimers

To further address the localization of $A_{2A}R/A_{2A}R$ dimers in CAD cells, $A_{2A}R$-CN/$A_{2A}R$-CC was co-transfected with four different fluorescent markers (Fig. 2A). Consistent with recent studies indicating that GPCR dimerization occurs at the ER [23–25], $A_{2A}R$-CN/$A_{2A}R$-CC fluorescence showed a moderate level of overlap with the ER marker (YFP-ER; YFP fused to the ER targeting sequence of calreticulin and the KEDL ER retrieval sequence) and with the transmedial Golgi marker (YFP-Golgi; YFP fusion to residues 1–81 of the 1,4-galactosyltransferase). Intracellular $A_{2A}R/A_{2A}R$ signals also co-localized with structures labeled with the endosomal marker RhoB [26] fused to YFP (YFP-Endo), suggesting trafficking of $A_{2A}R$ homodimers through early endosomes [27]. In the vast majority of the cells, distinct and robust cell surface signals were observed that co-localized with the membrane marker (mCherry-Mem, an N-terminal fragment of Gap43 fused to mCherry), suggesting efficient plasma membrane targeting of $A_{2A}R/A_{2A}R$ [12,22]. Because over-expression of fluorescently tagged markers may have influenced the expression and targeting of $A_{2A}R/A_{2A}R$, $A_{2A}R$-VN and $A_{2A}R$-VC were transfected in CAD cells in the absence of markers. Similar $A_{2A}R/A_{2A}R$ fluorescence patterns were observed in the presence and absence of the cellular markers (compare Fig. 2A and Supplementary

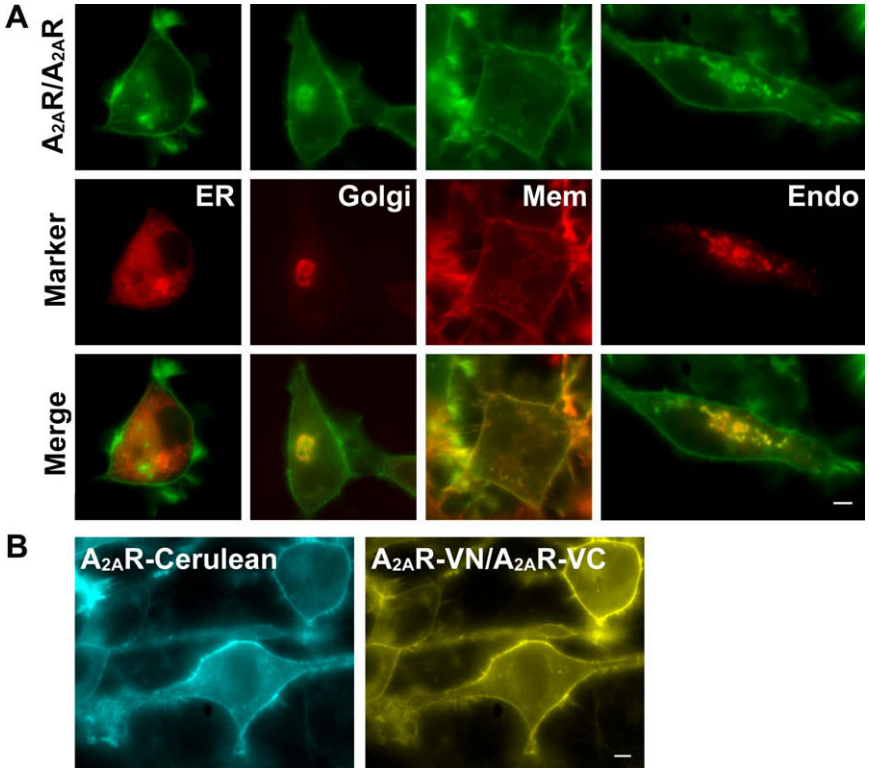

Fig. 2. Sub-cellular localization of $A_{2A}R/A_{2A}R$ oligomers. (A) CAD cells were co-transfected with $A_{2A}R$-CN and $A_{2A}R$-CC (in green), as well as YFP-Golgi, YFP-ER, mCherry-Mem, or YFP-Endo (in red). Overlapping signals in merged images appear yellow. (B) Cells expressing $A_{2A}R$-Cerulean, $A_{2A}R$-VN, and $A_{2A}R$-VC were analyzed by fluorescence microscopy. Cerulean signals and Venus complementation signals are shown in cyan and yellow, respectively. *Scale bars:* 5 μm.

Fig. 2). Virtually all cells displayed $A_{2A}R/A_{2A}R$ signals at the cell surface. Most cells displayed varying extents of $A_{2A}R/A_{2A}R$ in vesicular structures and in diffuse intracellular domains (ER), and some cells displayed clear golgi patterns. Cell-to-cell variations in the relative portion of ER, golgi, and vesicular labeling may reflect physiological differences or different states of cellular differentiation. In CAD cells expressing $A_{2A}R$-VN/$A_{2A}R$-VC and $A_{2A}R$-Cerulean, Venus and Cerulean signals overlapped (Fig. 2B), further indicating proper targeting of BiFC-tagged receptors. Fluorescence complementation signals were also detected in HEK293 cells transfected with $A_{2A}R$-VN and $A_{2A}R$-VC (Fig. 1C). However, the fluorescence patterns indicated reduced surface expression and increased ER retention of $A_{2A}R$-VN/$A_{2A}R$-VC in HEK293 cells compared to CAD cells. These observations suggest that neuronal factors in CAD cells may play a role in $A_{2A}R$ expression and targeting.

3.3. Higher-order $A_{2A}R$ oligomers detected by combined BiFC-FRET

Previous experiments have suggested the existence of higher-order GPCR oligomers containing $A_{2A}R$ receptors [14]. Thus, we used a novel experimental approach combining BiFC and FRET (Fig. 3A) to explore this possibility further. FRET is an established technique for studying protein–protein interactions in living cells that has been extensively applied to study GPCR dimerization (see [28] for review). FRET relies on the transfer of energy from a donor to an acceptor fluorophore occurring when both fluorophores reside in close vicinity (<10 nm). Several methods that measure changes in donor fluorescence intensity or lifetime have been developed to quantify FRET [29,30]. We first performed intensity FRET measurements with suspensions of cell expressing Cerulean (FRET donor) and Venus (FRET acceptor) fusion proteins. As shown in Fig. 3B, our system allowed us to discriminate between FRET signals from Cerulean–Venus concatemers with

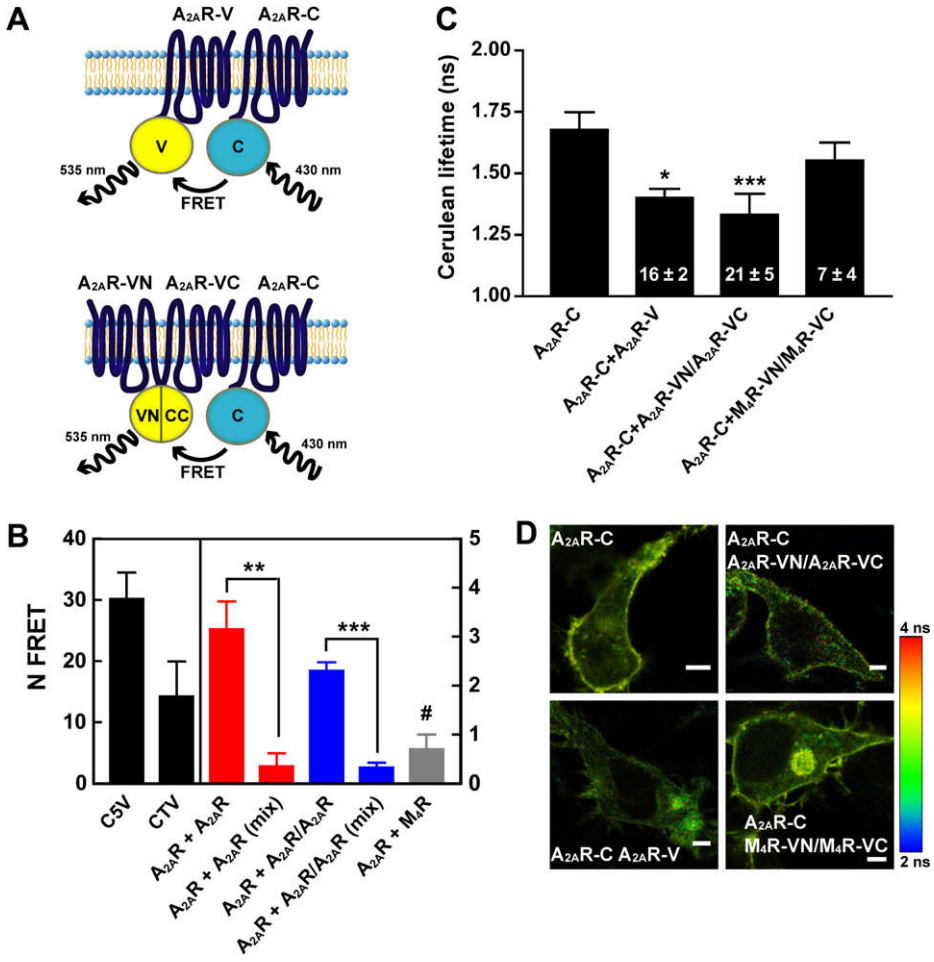

Fig. 3. Detection of $A_{2A}R$ higher-order oligomers by combining BiFC and FRET techniques. (A) Schematic representation of classical FRET (top) and BiFC-FRET (bottom) assays. Cerulean (C) or Venus (V) serve as donor and acceptor, respectively. In BiFC-FRET experiments, VN/VC fragments reconstitute Venus and serve as acceptor. (B) Intensity FRET measurements. CAD cells were transfected with $A_{2A}R$-Cerulean and $A_{2A}R$-Venus (red), $A_{2A}R$-VN + $A_{2A}R$-VC (blue), or M_4R-Venus as control (gray). Mix represents energy transfer in mixed populations of cells transfected solely with Cerulean or Venus fusions. #, $P < 0.05$ compared to $A_{2A}R + A_{2A}R$ or $A_{2A}R + A_{2A}R/A_{2A}R$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed unpaired *t*-tests, $n = 3$–4). The C5V and CTV concatemers of Cerulean and Venus were used to validate the FRET measurements. (C) FLIM-FRET measurements. CAD cells expressing $A_{2A}R$-Cerulean and $A_{2A}R$-Venus, $A_{2A}R$-VN + $A_{2A}R$-VC, or M_4R-VN + M_4R-VC were used for Cerulean fluorescence lifetime measurements. Data are means ± S.E.M. from at least five different image fields (15 cells). A representative experiment (out of three) is shown. FRET efficiency values (means ± S.E.M.) are indicated. *, $P < 0.05$; ***, $P < 0.001$ compared to $A_{2A}R$-C (ANOVA followed Dunnett's post hoc test). (D) Lifetime images from representative cells analyzed in C are shown together with the corresponding intensity scale. *Scale bars:* 5 μm.

distinct FRET efficiencies [31,32]. The C5V fusion construct in which Cerulean is connected to Venus by a 5 amino acid linker produced stronger FRET than the CTV construct, in which the fluorescent proteins are separated by 229 amino acids. FRET was detected in cells transfected with A_{2A}R-Cerulean and A_{2A}R-Venus, consistent with dimerization of A_{2A}R receptors (Fig. 3B). Cells expressing A_{2A}R-Cerulean, A_{2A}R-VN, and A_{2A}R-VC also displayed FRET signals, suggesting the existence of A_{2A}R oligomers with more than two protomers. Very low signals were obtained from mixed cell populations (expressing either Cerulean or Venus fusions). Moreover, significantly lower energy transfer was observed in control experiments where the M_4R receptor replaced A_{2A}R as acceptor. To further confirm the intensity FRET data, fluorescence lifetime measurements were undertaken. In contrast to intensity-based FRET measurements which are complicated by the necessity of correcting for fluorescence cross-talk and bleed-through resulting from spectral overlap between donor and acceptors, FLIM-FRET relies on donor lifetime measurements and hence represents an independent method to quantify FRET. Decreased Cerulean lifetime indicative of FRET was measured in cells expressing A_{2A}R-Cerulean and A_{2A}R-Venus or A_{2A}R-Cerulean, A_{2A}R-VN, and A_{2A}R-VC (Fig. 3C and D) but not in transfections with A_{2A}R-Cerulean, M_4R-VN, and M_4R-VC. Venus fluorescence was detected in cells expressing M_4R-VN/M_4R-VC and A_{2A}R-Cerulean (Supplementary Fig. 1B), indicating that the absence of energy transfer in A_{2A}R-Cerulean/M_4R-VN/M_4R-VC transfections was not caused by the absence of an acceptor. When regions of interest only comprising cell surface were analyzed, very similar Cerulean lifetimes were measured (Supplementary Fig. 3). Together, these results highlight the propensity of A_{2A}R to homo-dimerize and further indicate that A_{2A} receptors assemble into higher-order oligomers at the plasma membrane.

4. Discussion

Although GPCRs have been proposed to associate as higher-order oligomers (“mosaics”) at the plasma membrane [3], and a growing number of GPCR–GPCR interactions are being reported [5], most classical techniques are restricted to the detection of two interacting proteins, leaving open the possibility that (at least some) GPCRs form “alternative dimers”. Only recently has the development of new, non-invasive, approaches allowed scientists to probe for higher-order GPCR complexes. By implementing a three-chromophore FRET (3-FRET) protocol [33], Lopez-Gimenez and colleagues [34] showed that recombinant α_{1b}-adrenoreceptors exist as higher-order oligomers in HEK293 cells. Moreover, with the development of a new technique in which sequential bioluminescence resonance energy transfer (BRET) and FRET (SRET) detection serves as a readout for trivalent protein complexes, Carriba et al. [35] have demonstrated hetero-oligomerization of A_{2A}, D_2 and cannabinoid CB_1 receptors. Finally, a combination of BRET and BiFC was used to identify ternary complexes between calcitonin receptor-like receptors and the receptor activity-modifying protein 1 [36].

A related technique combining BiFC and FRET for the detection of trivalent protein complex has been recently developed [37]. While BRET assays (as well as combined assays using BRET) are used to assess the affinity and specificity of protein–protein interactions [38], BiFC-FRET, when measured using the standard 3-cube method [39] or with FLIM, allows not only the detection of ternary complex formation, but also the identification of the sub-cellular localization of the interaction. Moreover, in contrast to the 3-FRET technique which also has spatial resolution, BiFC-FRET does not require sophisticated filter combinations and data processing. Because of the irreversible nature of BiFC [15], it should, however, be emphasized that the technique is likely to stabilize the formation and to facilitate the detection of oligomeric partners.

In this report, we used BiFC-FRET to probe for higher-order GPCR oligomers in the neuronal CAD cell model. The data demonstrate the assembly of A_{2A}R in higher-order oligomers. While preparing this manuscript, a report by Gandia et al. [40] was published in which trivalent A_{2A}R complexes were detected with the BiFC-BRET assay: co-expression of A_{2A}R fusions to N- and C-terminal fragments from the yellow fluorescent protein and to *Renilla* luciferase resulted in saturable BRET signals indicative of trimeric A_{2A}R complexes. Our data confirm and expand these findings by demonstrating the existence of higher-order A_{2A}R oligomers at the plasma membrane, the recognized site of action of A_{2A}Rs. In addition to forming homomers, A_{2A} receptors are known to associate in heteromeric complexes, notably with adenosine A_1 [22,41], dopamine D_2 [11–14], cannabinoid CB_1 [42], and glutamate $mGlu_5$ [43] receptors. Future experiments using the BiFC-FRET technique described here will investigate higher-order GPCR heteromers. We have recently shown that prolonged A_{2A}R or D_2R stimulation affects the proportion of receptors engaging in homodimers and heterodimers [14] and of particular interest will be the analysis of drug effects on higher-order GPCR assemblies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.062.

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